

COMPARISON OF VARIOUS LAZAROID COMPOUNDS FOR PROTECTION AGAINST ISCHEMIC LIVER INJURY^{1,2}

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Lazaroids are a group of 21-aminosteroids that lack steroid action but have a potent cytoprotective effect by inhibiting iron-dependent lipid peroxidation. However, there have been conflicting reports on the effectiveness and potency of the various lazaroïd compounds. In this study, we compared the effectiveness of three major lazaroïd compounds on warm liver ischemia in dogs using a 2-hr hepatic vascular exclusion model. The agents were given to the animals intravenously for 30 min before ischemia. The animals were divided into 5 groups: Control (n=10), no treatment; Group F (n=6), U-74006F (10 mg/kg); Group G (n=6), U-74389G (10 mg/kg); Group A1 (n=6), U-74500A (10 mg/kg); Group A2 (n=6), U-74500A (5 mg/kg). The effect of treatment was evaluated by two-week animal survival, hepatic tissue blood flow, liver function tests, blood and tissue biochemistry, and histological analyses. Animal survival in all treated groups was significantly improved compared with the control (83–100% versus 30%). Elevation of liver enzymes after reperfusion was markedly attenuated in treated groups, except for an early significant increase in Group G. Postreperfusion hepatic tissue blood flow was much higher in all treated animals (50% of the preischemic level vs. 25% in the control). Lazaroids, particularly U-74500A at 5 mg/kg (Group A2), suppressed adenine nucleotide degradation during ischemia and enhanced the resynthesis of high-energy phosphates after reperfusion. Although structural abnormalities in postreperfusion liver tissues were markedly ameliorated in all treated groups, Group A2 showed significantly less neutrophil infiltration. Liver injury from warm ischemia and reperfusion was attenuated with all lazaroïd compounds, of which U-74500A at 5 mg/kg exhibited the most significant protective activity.

Lazaroids, 21-aminosteroids lacking the negative aspect of steroid activity, have cytoprotective properties against iron-dependent lipid peroxidation (1). In addition to antioxidant properties, lazaroïds also exert their cytoprotective affect by inhibition of arachidonic acid release (1), stabilization of cell membranes (2), suppression of Kupffer cell activation (3), and down-regulation of cytokine expression and release (4, 5). The efficacy of lazaroïds in preventing ischemia and reperfusion injury or extending organ preservation for various organs and animal species has been reported by us (6) and

others (7–9). However, there have been conflicting reports on the potency of the various lazaroïd compounds (10–12). Three major lazaroïd compounds, U-74006F, U-74389G, and U-74500A were tested in this study to determine which was the most effective in abating ischemia and reperfusion injury of canine livers subjected to 2-hr of warm ischemia using a total hepatic vascular exclusion model.

MATERIALS AND METHODS

Animals. Adult female beagle dogs, weighing 8.0 to 12.8 kg, were used for this study. The experiment was performed with the approval of the Animal Care and Use Committee of the University of Pittsburgh, and were managed in accordance with guidelines issued by the National Institutes of Health and the Public Health Service Policy on the humane use and care of laboratory animals. After an overnight fast, the animals were anesthetized with 25 mg/kg of intravenous thiopental-sodium (Abbott Laboratories, North Chicago, IL), intubated, and maintained with a mixture of isoflurane, oxygen, and nitrous oxide by positive mechanical ventilation. The right carotid artery and the right jugular vein were cannulated for monitoring of arterial blood pressure and central venous pressure, and for serial blood sampling. Electrocardiogram, arterial blood gas, electrolytes, and esophageal temperature were also monitored throughout the operation.

Operative procedures. Through a midline incision, the liver was completely skeletonized by dividing all the hepatic suspensory ligaments and by dissecting the retrohepatic vena cava from the posterior abdominal wall. Before starting total hepatic venous exclusion, venovenous bypass (Biomedicus, Minnetonka, MN), connecting the femoral vein, the splenic vein and the left jugular vein via Tygon tubing (Norton Industrial Plastics, Akron, OH), was used to decompress the splanchnic venous beds and the infrahepatic vena cava. Heparin sodium (Upjohn, Kalamazoo, MI), 50 U/kg, was administered intravenously 5 min before hepatic ischemia to prevent thrombus formation. Ischemia was begun by totally excluding the vasculature of the liver. This was accomplished by cross-clamping the portal vein and the hepatic artery (together with the hepatoduodenal ligament) and the inferior vena cava above and below the liver. Total hepatic vascular exclusion was maintained for 2 hr, after which ischemia was stopped by opening the clamps and removing the bypass system. A splenectomy was performed immediately after the ischemic period.

During the operation, an electrolyte solution (Plasmalyte, Baxter, Deerfield, IL) was continuously infused to the animals at a rate of 20 ml/kg/hr. Sodium bicarbonate and dopamine hydrochloride were given, if necessary, to maintain base excess above –3.0 and systolic blood pressure above 90 mmHg. Cephmandole nafate (1 g) was given intraoperatively, and continued daily for 3 days. Animals were allowed to eat and drink from the next morning.

Experimental groups. The lazaroïd compounds and citrate buffer (pH 3.0) were supplied by the Upjohn Company (Kalamazoo, MI). The lazaroïds were dissolved in the citrate buffer vehicle at a concentration of 1.5 mg/ml with U-74006F, and at 2 mg/ml with U-74389G and U-74500A. Animals were divided into five groups:

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Control (n=10, no treatment), Group F (n=6, 10 mg/kg of U-74006F), Group G (n=6, 10 mg/kg of U-74389G), Group A1 (n=6, 10 mg/kg of U-74500A), and Group A2 (n=6, 5 mg/kg of U-74500A). Lazaroids were given as a continuous intravenous infusion for 30 min before ischemia. Animals were followed for two weeks after liver ischemia.

Determinations. The cytoprotective effect of the respective lazaroid compound was studied using the following determinants: two-week animal survival, liver function tests, hepatic tissue blood flow, blood and tissue biochemistry, and histological analyses.

Indocyanine green retention test: The effectiveness of hepatic vascular exclusion was determined using the indocyanine green (ICG)* retention test. Indocyanine green (ICG, Cardio-Green, Beckton Dickinson Microbiology Systems, Cockeysville, MD) at a dose of 0.5 mg/kg, was intravenously administered 30 min after the onset of total hepatic venous exclusion, and 3 ml of heparinized arterial blood was collected 20 min after ICG injection. Indocyanine green retention rate was determined using a spectrophotometer (Lamda, Perkin Elmer, Norwalk, CT) at a wavelength of 805 nm.

Hepatic tissue blood flow: Tissue blood flow, at the hepatic surface, was measured using a laser-Doppler flow meter (Advance laser flowmeter, ALF21, Advance Company Ltd., Tokyo, Japan) before ischemia, during ischemia, and 5 min, 15 min, 30 min, and 60 min after reperfusion. Tissue blood flow was calculated as the mean flow of the right hepatic lobe, the middle hepatic lobe, and the left hepatic lobe. Tissue flow is expressed as a percentage of the preischemic level.

Liver function tests: Blood samples for liver function tests were collected before ischemia, after 2 hr of ischemia, and 15 min, 60 min, 3 hr, 6 hr, 12 hr, 24 hr, 2 days, 3 days, 5 days, 7 days, 10 days, and 14 days postischemia. A Technicon RA500 autoanalyzer (Bayer, Tarrytown, NY) was used to determine plasma levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), and total bilirubin.

Plasma malondialdehyde: Heparinized arterial blood samples were collected before ischemia, at the end of ischemia, and 15 min and 60 min after reperfusion. Plasma malondialdehyde levels were measured according to the method by Yagi (13).

Tissue biochemistry: A small wedge biopsy was taken from the liver before ischemia, 1 hr and 2 hr after onset of ischemia, and 15 min and 60 min after reperfusion. The tissue samples were immediately (5–10 secs) frozen in liquid nitrogen and stored at -80°C until analysis. Tissue concentrations of adenine nucleotides (AN) and purine catabolites (PC) were measured using a Waters HPLC system (Waters Chromatography Division, Millipore Corp., Milford, MA; Model 510 pumps, Model 484 absorbance module, and Model 717 WISP system), equipped with a tunable absorbance detector (Waters Model 484). Energy charge (EC) was calculated using the equation (14) $\text{ATP} + 0.5 \text{ADP} / (\text{ATP} + \text{ADP} + \text{AMP})$.

Histopathology: A small portion of each tissue taken for tissue biochemistry was fixed in buffered formalin, paraffin-embedded and stained with hematoxylin eosin. Histopathologic changes were blindly read by a single pathologist. Neutrophil infiltration was determined by taking the mean count made in twenty randomly selected observation fields (at a magnification of $100\times$) of each section/animal after neutrophil staining by Leder's method.

Statistics. Values are expressed as mean \pm SEM. Animal survival was determined using the chi-square test. Intragroup analysis was performed using the paired Student's *t* test. Intergroup analysis was performed using ANOVA. When the analysis of variance showed a

significant difference ($P < 0.05$), a post hoc test was used to determine the *P* values for each group.

RESULTS

Clinical observations. Although mean arterial blood pressure (MAP) of the Control dogs showed a slight decline soon after initiating hepatic venous exclusion (from 113.25 ± 6.01 mmHg to 95.05 ± 8.46 mmHg), the MAP remained relatively stable (>100 mmHg) during liver ischemia, with a mean bypass flow of 0.85 ± 0.05 L/min. After reperfusion, however, all animals developed hypotension (52.11 ± 5.65 mmHg at 5 min after reperfusion). The blood pressure gradually recovered to preoperative levels 3 hr after reperfusion. During the 30 min intravenous drug infusion, U-74006F and U-74389G caused no significant hemodynamic changes, but moderate and significant hypotension occurred in Group A1 animals, with 4 animals developing arrhythmia. Decrease of the U-74500A dose from 10 mg/kg (Group A1) to 5 mg/kg (Group A2) abrogated hypotension and arrhythmia.

Liver revascularization in the control animals was characterized by severe swelling, mottled reperfusion, and a palpably hard liver, all indicating an outflow block. These characteristics were seen in only one animal of Group F and Group G, and none in Group A1 or Group A2.

ICG retention rate. The completeness of hepatic venous exclusion was confirmed by ICG retention rate. Indocyanine green retention rate during liver ischemia was $94.82 \pm 1.26\%$ in the controls, $96.61 \pm 2.64\%$ in Group F, $95.18 \pm 0.69\%$ in Group G, $96.02 \pm 1.77\%$ in Group A1, and $95.98 \pm 1.83\%$ in Group A2. There were no significant differences between the groups.

Survival. Survival of the treated groups was significantly better than Control. By the end of the two-week follow-up period, seven of ten control animals died from liver failure, six on postoperative day (POD) 1 and another on POD 10. In contrast, all but one animal in the treated groups survived for two weeks; one of the Group F animals died on POD 2 from bleeding due to an injured splenic vein with normalizing liver enzymes. There was no statistically significant difference in animal survival between lazaroid groups.

Liver function tests. The LDH of the Control animals remained high for 12 hr after reperfusion (Fig. 1A). Among the lazaroid-treated groups, Group G showed a much higher elevation in LDH than the Control 15 minutes after reperfusion, while the other three groups had lower LDH levels than the Control throughout the observation period. Aspartate aminotransferase and ALT (Fig. 1B) of the Control animals elevated rapidly after unclamping, reaching the maximal values 12 hr after reperfusion. Transaminase levels of Group F, Group A1, and Group A2 were significantly lower than the Control and Group G during the early postoperative period. There was no statistically significant difference in LDH, AST, or ALT levels between Groups F, A1, and A2. Two animals in the Control group had a total bilirubin level greater than 3 mg/dl; one died at POD 1 from liver failure and the other lived for 14 days. In the lazaroid groups, hyperbilirubinemia was seen in only one Group G animal.

Hepatic tissue blood flow. During ischemia, hepatic tissue blood flow decreased to $10.40 \pm 1.33\%$ of the preischemic level in all groups. After reperfusion, restoration of blood flow was significantly inhibited in the Control group ($23.9 \pm 5.7\%$ 60 min after reperfusion). However, lazaroid treatment aug-

* Abbreviations: ADP, adenosine diphosphate; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AMP, adenosine monophosphate; AN, adenine nucleotides; AST, aspartate aminotransferase; ATP, adenosine triphosphate; EC, energy charge; ICG, indocyanine green; LDH, lactate dehydrogenase; MAP, mean arterial pressure; MDA, plasma malondialdehyde; PC, purine catabolites; POD, postoperative day.

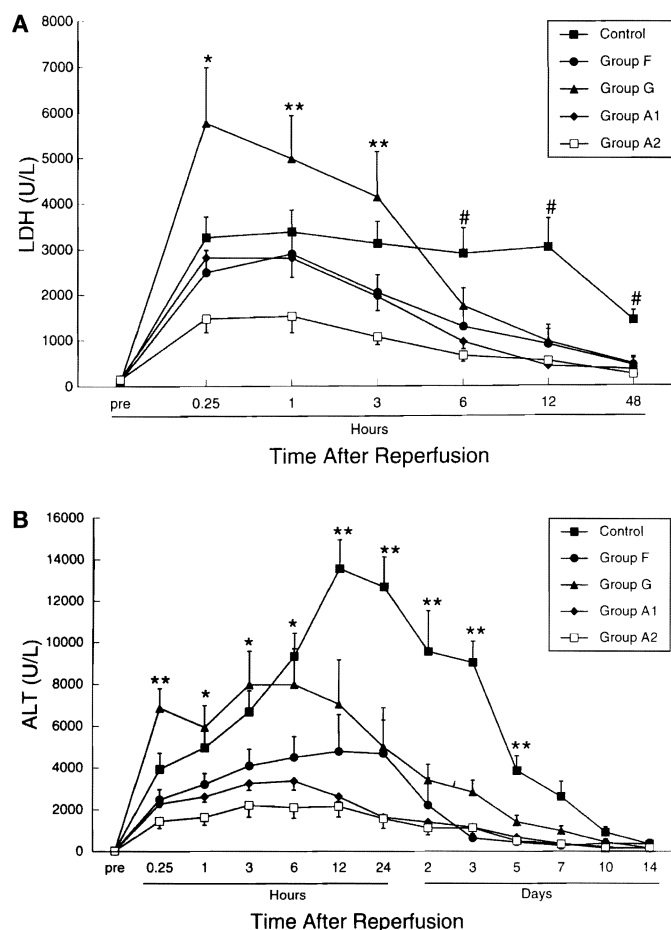


FIGURE 1. (A) Changes in lactate dehydrogenase (LDH) after reperfusion: * $P < 0.05$ vs. Control, Groups F, A1, A2; ** $P < 0.05$ vs. Groups F, A1, A2; # $P < 0.05$ vs. Groups F, G, A1, A2. (B) Changes in alanine aminotransferase (ALT) after reperfusion: * $P < 0.05$ vs. Groups F, A1, A2; ** $P < 0.05$ vs. Groups F, G, A1, A2.

mented postreperfusion blood flow to more than 40% in all groups. Group A2 showed markedly higher hepatic tissue blood flow than the other three lazardoid groups (Fig. 2).

Plasma MDA levels. There was no significant difference in plasma MDA levels between Control and lazardoid groups at the end of two-hour liver ischemia (Fig. 3). Reperfusion caused a significant increase in MDA in all groups; however, the increase in plasma MDA of Group A2 15 and 60 min after reperfusion was significantly lower than Control.

Energy metabolism. Changes in adenine nucleotides and purine catabolites, reflecting energy metabolism in the hepatic tissue, during ischemia and after reperfusion are shown in Table 1. Hepatic ischemia induced marked degradation of ATP, resulting in higher concentrations of AMP and purine catabolites. After reperfusion, energy re-synthesis (estimated by energy charge) was poor 15 min after reperfusion, but recovered to normal levels at 60 min. Compared to Control, ATP degradation during hepatic ischemia was moderately suppressed by the treatment with lazardoids. In fact, U-74500A at 5 mg/kg (Group A2) showed significantly better energy charge, higher adenine nucleotide levels, and less accumulation of purine catabolites than Control. In addition, Group A2 animals showed a prompt restoration of energy charge to normal levels 15 min after reperfusion, while en-

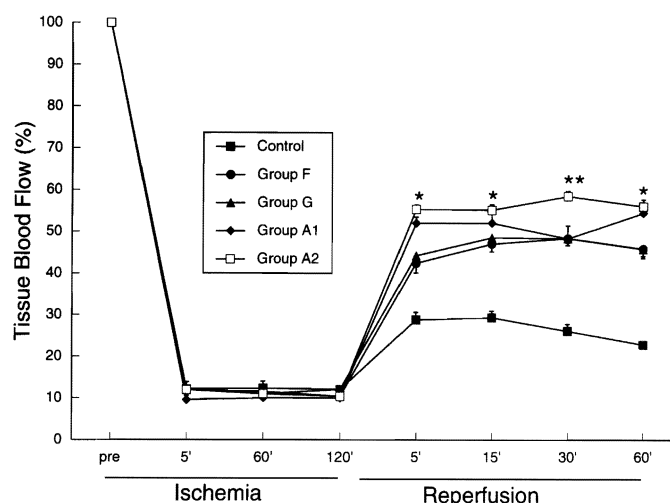


FIGURE 2. Tissue blood flow in the liver during ischemia and after reperfusion. Values are expressed as a percentage of the preischemic level. * $P < 0.05$ vs. Control, Groups F, G; ** $P < 0.05$ vs. Control, Groups F, G, A1.

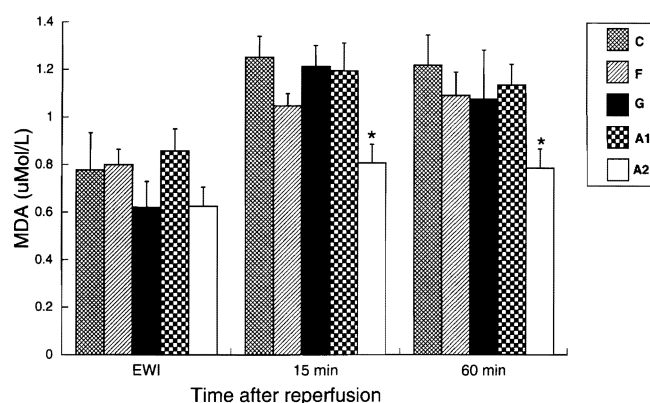


FIGURE 3. Changes in plasma malodialdehyde (MDA) at the end of warm ischemia and after reperfusion. * $P < 0.05$ vs. Control.

ergy charge of the other three lazardoid groups remained poor. As with the Control, energy charge returned to normal levels 60 min after reperfusion in all lazardoid groups, except for Group G.

Histopathology. The 2-hr total liver ischemia caused no remarkable changes in liver structure except for occasional single hepatocyte necrosis that was seen equally in all groups. Although the basic architecture was preserved, Control livers developed marked abnormalities after reperfusion, such as centrilobular hepatocyte necrosis, ballooning, degeneration, and hemorrhagic congestion (Fig. 4A). Detachment of sinusoidal cells was found frequently in the vicinity of necrotic areas, accompanied by intense neutrophil infiltration. Lazardoids prevented sinusoidal congestion and inhibited hepatocyte necroses and ballooning. Only a few neutrophils were seen in a scattered area (Fig. 4B). In comparing neutrophil infiltration 60 min after reperfusion, the number counted in Group A2 was significantly less than all other groups (Fig. 5).

TABLE 1. Biochemistry of liver tissues

| Groups | EC ^a (nmol/mg protein) | TAN (nmol/mg protein) | ATP (nmol/mg protein) | ADP (nmol/mg protein) | AMP (nmol/mg protein) | HX (nmol/mg protein) | XT (nmol/mg protein) |
|------------------------------|--------------------------------------|-----------------------------|---------------------------------|----------------------------|----------------------------|------------------------------|----------------------------|
| Normal | 0.828±0.016 | 30.3972±2.1550 | 21.771±2.036 | 6.9079±0.7475 | 1.71885±0.25702 | 0.8385±0.1160 | 0.05963±0.03282 |
| At 1 hr warm ischemia: | | | | | | | |
| Control | 0.301±0.006 | 25.324±4.096 | 4.180±0.541 | 6.779±1.241 | 14.365±2.360 | 2.893±0.338 | 2.807±0.214 |
| Laz F | 0.294±0.014 | 23.382±3.008 | 4.014±0.788 | 6.126±0.851 | 13.242±1.402 | 3.350±0.473 | 2.928±0.317 |
| Laz G | 0.399±0.032 | 24.747±2.051 | 6.288±1.562 | 6.869±0.602 | 11.621±2.373 | 2.321±0.409 ^c | 1.566±0.558 ^{b,c} |
| Laz A1 | 0.393±0.021 | 21.699±1.899 | 5.261±0.624 | 6.656±0.859 | 9.782±0.747 ^b | 1.859±0.209 ^{b,c} | 1.083±0.120 ^{b,c} |
| Laz A2 | 0.454±0.032 ^{b,c} | 20.474±2.590 | 6.064±0.955 | 6.370±0.924 | 8.040±1.268 ^{b,c} | 0.873±0.269 ^{b,c,d} | 0.827±0.208 ^{b,c} |
| At 2 hr warm ischemia: | | | | | | | |
| Control | 0.204±0.028 | 12.516±0.857 | 1.384±0.278 | 2.375±0.233 | 8.838±0.612 | 3.483±0.244 | 5.438±0.418 |
| Laz F | 0.309±0.019 ^b | 15.674±1.520 | 2.903±0.335 ^b | 3.873±0.505 | 8.898±0.905 | 3.337±0.447 | 4.113±0.681 |
| Laz G | 0.254±0.016 | 14.402±1.265 | 2.082±0.154 | 3.230±0.318 | 9.296±1.019 | 2.036±0.316 ^{b,c} | 3.054±0.394 ^b |
| Laz A1 | 0.278±0.035 | 14.923±1.332 | 2.408±0.489 | 3.686±0.555 | 8.820±0.832 | 2.495±0.452 | 3.528±0.635 ^b |
| Laz A2 | 0.317±0.029 ^b | 15.961±1.464 | 3.418±0.584 ^{b,d} | 3.571±0.548 | 8.972±0.626 | 1.638±0.171 ^{b,c} | 2.844±0.511 ^b |
| At 15 min after reperfusion: | | | | | | | |
| Control | 0.723±0.032 | 12.053±2.105 | 7.240±1.468 | 3.541±0.665 | 1.324±0.150 ^c | 1.101±0.212 | 0.418±0.229 |
| Laz F | 0.709±0.047 | 14.632±0.586 | 8.451±0.822 | 3.748±0.451 | 2.433±0.538 | 0.919±0.184 | 0.065±0.028 ^b |
| Laz G | 0.716±0.021 | 15.170±1.796 | 8.783±1.194 | 4.360±0.570 | 2.060±0.275 | 1.446±0.414 | 0.049±0.028 ^b |
| Laz A1 | 0.752±0.034 | 13.928±0.692 | 8.788±0.687 | 3.406±0.353 | 1.734±0.412 | 0.722±0.134 ^d | 0.034±0.014 ^b |
| Laz A2 | 0.831±0.013 ^{b,c,d} | 16.648±0.929 ^b | 12.409±0.827 ^{b,c,d,e} | 2.903±0.122 | 1.337±0.187 ^c | 0.693±0.159 ^d | 0.025±0.025 ^b |
| At 60 min after reperfusion: | | | | | | | |
| Control | 0.832±0.014 | 12.175±0.907 | 8.934±0.758 | 2.417±0.266 | 0.823±0.097 | 0.776±0.145 | 0.257±0.056 |
| Laz F | 0.858±0.006 ^d | 14.774±0.608 | 11.479±0.552 | 2.435±0.109 | 0.861±0.034 | 0.715±0.130 | 0.235±0.045 |
| Laz G | 0.734±0.08 | 20.557±3.359 ^{b,c} | 11.744±1.938 | 4.820±0.998 ^{b,c} | 1.396±0.150 | 0.838±0.076 | 0.508±0.261 |
| Laz A1 | 0.82±0.008 | 18.501±1.028 ^b | 13.028±0.846 ^b | 3.948±0.326 | 1.525±0.127 | 0.945±0.109 | 0.368±0.067 |
| Laz A2 | 0.842±0.012 | 16.745±1.449 | 12.591±1.274 ^b | 3.123±0.219 | 1.031±0.174 | 0.352±0.188 ^{b,d,e} | 0.012±0.012 ^d |

^a EC: energy charge; TAN: total adenosine nucleotide; ATP: adenine nucleotide; HX: hypoxanthine; XT: xanthine.^b P<0.05 versus Control.^c P<0.05 versus Group F.^d P<0.05 versus Group G.

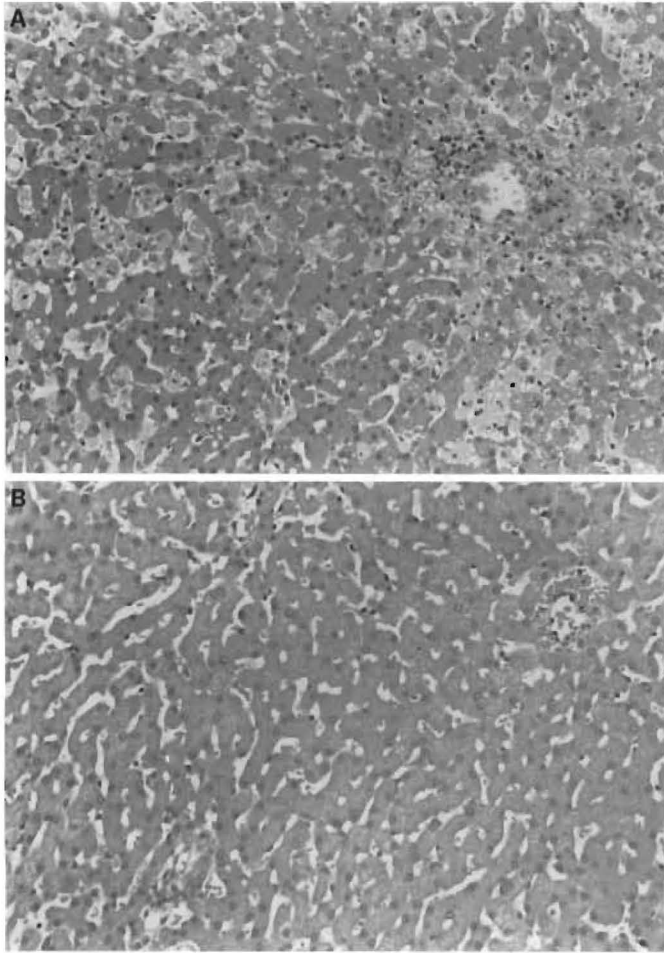


FIGURE 4. (A) Control group liver tissue 60 min after reperfusion. Hepatocytes necrosis and vacuolation were found at pericentral area and other scattered areas. Hemorrhage and sinusoidal congestion were found at the site of parenchymal necrosis. Parenchymal damage was associated with neutrophil infiltration. (H&E; $\times 200$). (B) Group A2 liver tissue 60 min after reperfusion. Liver architecture was well preserved. Liver parenchymal damage was not significant, with only single cell necrosis or vacuolation. Hemorrhage and sinusoidal congestion were rare. Neutrophil infiltration was reduced compared to Control. (H&E; $\times 200$).

DISCUSSION

This study showed that prolonged hepatic warm ischemia causes severe liver damage and high mortality in control animals. In contrast, administration of lazaroid compounds before ischemia attenuated ischemic liver damage and improved animal survival. In addition, we showed that U-74500A, at a dose of 5 mg/kg, was most effective compared to the other lazaroid compounds, allowing better hepatic tissue blood flow, lower liver enzyme elevation, improved energy metabolism, less lipid peroxidation, and reduced neutrophil accumulation.

In this study, we used a total hepatic vascular exclusion model to examine the effectiveness of lazaroids against liver ischemia. Most previous studies of warm liver ischemia and reperfusion have used Pringle's maneuver (15, 16), which interrupts the hepatic blood in-flow at the hepatic hilum. When the mesenteric venous beds are appropriately decompressed by a passive or pump-driven portasystemic bypass

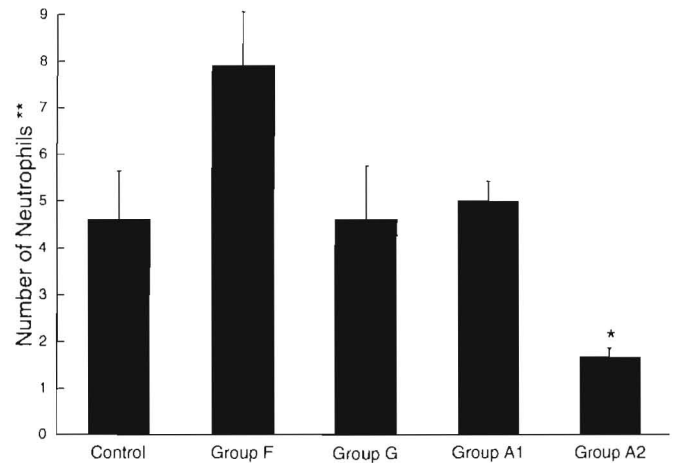


FIGURE 5. Number of neutrophils in the liver 60 min after reperfusion: * $P < 0.05$ vs. Control, Groups F, G, A1; ** Average numbers in 10 power fields ($\times 100$).

(17), dogs have been shown to tolerate liver ischemia for 60 min (18), 90 min (19), or 180 min (17). The total hepatic vascular exclusion model causes more severe liver ischemia than the Pringle's maneuver, due to the almost total absence of hepatic blood flow. While the Pringle's maneuver occludes only the portal vein and hepatic artery, total hepatic vascular exclusion also occludes the infrahepatic and suprahepatic vena cava. The effectiveness of the total hepatic vascular exclusion model was confirmed by measuring the ICG retention rate, which was more than 95% during ischemia. Using the total hepatic vascular exclusion model, we first showed that 30% of the control dogs could tolerate two hours of hepatic warm ischemia. This result is in direct contrast to other reports (20, 21) showing mortality in all animals subjected to 60 min of hepatic vascular exclusion. The animal strain and perioperative management, including careful maintenance of bypass flow and fluid and electrolyte balance, might be responsible for the difference in results.

There appeared to be significant differences in the effectiveness of the three lazaroid compounds at the same 10 mg/kg dose. The 10 mg/kg dose was selected based on our previous study, in which 10 mg/kg of intravenous U-74389G administered to the donors 30 min before procurement was more effective than doses of 6 mg/kg or 15 mg/kg in 48-hr canine liver preservation and transplantation. At the 10 mg/kg dose, U-74500A was more effective than U-74006F and U-74389G. Group G had much higher liver enzymes during the early postoperative period compared with Group A and Group F. Lazaroid U-74500A significantly reduced accumulation of purine catabolites in the ischemic liver tissue, and enhanced hepatic tissue blood flow after reperfusion compared with Lazaroid F. In addition, when the dose of U-74500A was decreased to 5 mg/kg (Group A2), it not only eliminated cardiovascular adverse effects that were frequently seen with 10 mg/kg of U-74500A (Group A1), but also allowed better hepatic tissue blood flow, less MDA production, prompt energy resynthesis, and better hepatic structure than Group A1 animals. The different protective activity between Groups A1 and A2 may result from a U-shaped dose-dependent effectiveness (22), which seems to be inherent to the lazaroid compounds. This U-shaped dose-response

curve was observed in this study and in our previous liver preservation experiment with U-74389G (6).

The differing potency of lazaroids determined in our experiment may be related to the site of action of the respective agents in the lipid peroxidation cascade. Lipid peroxidation is caused by oxygen radicals, which are generated as superoxide anions by the conversion of hypoxanthine to xanthine during reoxygenation. Mitochondrial oxidative phosphorylation, arachidonic acid metabolism, and the NADPH-dependent oxidase system on the surface membrane of neutrophils are other sources of superoxide radical generation. Once generated, superoxide anions propagate to produce hydroxyl radicals by Haber-Weiss reaction and Fenton reduction with participation of ferrous iron. Hydroxyl radicals attack unsaturated fatty acids to form lipid peroxy radicals, which proceeds to a radical-driven chain reaction causing more peroxidation of unsaturated fatty acids. Although all three lazaroid compounds are potent antioxidants, U-74500A is 2–10 times more effective in inhibiting iron-dependent lipid peroxidation, by chelating iron, than U-74006F (23)—however, U-74006F and U-78517G are more effective in scavenging lipid peroxy radicals (like α -tocopherol) than U-74500A (1, 24). Thus, the potency of U-74500A, as shown in our experiment, might indicate that early inhibition of lipid peroxidation by iron chelation is more beneficial than scavenging lipid peroxy radicals that have already formed. Killinger *et al.* (25) reported that U-74500A was more potent than U-75412E and U-74006F for preservation of endothelial cell viability in tissue culture and cold storage.

At aerobic conditions, cellular function is regulated by the level of high-energy phosphate (ATP) produced exclusively by oxidative phosphorylation in the mitochondria. When tissue becomes ischemic, ATP is progressively degraded to other adenine nucleotides, nucleosides, and finally, purine catabolites. Levels of ATP are restored after reoxygenation via the salvage pathway and through the slow, but more efficient, pathway of *de novo* synthesis. Pre-ischemic treatment with lazaroids, particularly U-74500A, slowed ATP degradation, suppressed PC accumulation, and enhanced ATP resynthesis. Although the mechanism is unclear, the beneficial effect of lazaroids in ischemic and postischemic energy metabolism appears to be related to their antioxidant activity. While most studies of lipid peroxidation have focused on events after reoxygenation, only a few studies have examined the events occurring during ischemia, and these have revealed that lipid peroxidation also occurs to some extent during ischemia (19, 26). Once cellular membranes are damaged by free radicals attack during ischemia, they become leaky to constituents of adenine nucleotides (27). The adenine nucleotides are converted to nucleosides in the interstitial space, and taken up by endothelial cells (28), where they are catabolized to hypoxanthine. Since sinusoidal endothelial cells are abundant in xanthine oxidase (29), they may be more vulnerable to oxygen radicals generated by the xanthine oxidase system when the blood supply is restored. It has been previously shown in rat livers that lipid peroxidation occurs in the endothelial cells during warm ischemia (30). Endothelial cell protection should lead to the preservation of the hepatic microcirculation. The early resumption of energy metabolism and higher tissue blood flow associated with lazaroid treatment in this study, and as described previously by us (6) and others (7, 8), suggests that lazaroids protect not only the

parenchymal hepatocytes, but the sinusoidal endothelial cells as well.

There is a growing body of evidence demonstrating that neutrophils are vigorously involved in ischemia and reperfusion injury of organs. Neutrophil depletion and the administration of antineutrophil serum or antineutrophil monoclonal antibody significantly reduced damage in the heart (31), liver (32), and small bowel (33). Neutrophils accumulate, adhere, and are activated in postischemic liver tissues under the regulation of cytokines, chemotactic factors, and adhesion molecules that are produced by Kupffer cells, endothelial cells, hepatocytes, and inflammatory cells in the liver. After reperfusion, activated Kupffer cells produce inflammatory cytokines, interleukin-1 and tumor necrosis factor, that activate endothelial cells and neutrophils. These cytokines, as well as leukotriene and platelet activating factor produced via arachidonic acid metabolism, have a chemotactic property for neutrophils. In addition, the cytokines upregulate expression of adhesion molecules on the surface of neutrophils to facilitate their rolling, adhesion, and activation. Activated neutrophils not only generate superoxide anions but also release myeloperoxidase and elastase, which further augment the destruction of liver tissues. Lazaroids have been shown to interrupt the chain of the inflammatory cascade that leads to neutrophil activation by affecting Kupffer cells (3) and cytokines (4), leukotrienes (34), oxygen radicals (35), and neutrophil enzymes (34). In the present study, U-74500A at 5 mg/kg showed less neutrophil infiltration in postischemic liver tissues.

In conclusion, lazaroids are cytoprotective to the canine liver during 2 hr of warm liver ischemia. Lazaroid U-74500A at a dose of 5 mg/kg was found most effective. Although lazaroids are very effective in the amelioration of ischemia organ damage, the hydrophobic nature of the compounds may limit their practical applicability. One solution would be the development of a new agent that has similarly potent antioxidant activity with hydrophilic properties, allowing oral administration and dissolution in the organ preservation fluid, which is currently underway.

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